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Rapid Upregulation of the Pi Isoform of Glutathione-*S*-Transferase in Mouse Brains After Withdrawal of the Neurotoxicant, Cuprizone

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ABSTRACT

Cuprizone intoxication has been used as a model for reversible demyelination in the CNS. During the course of cuprizone intoxication, the glutathione-*S*-transferase isoform, Pi, normally an oligodendrocytic marker, appears in reactive astrocytes (Cammer and Zhang, 1993). The present experiments address the changes in expression of Pi after removal of cuprizone from the diet of the affected mice. In order to localize Pi message, a riboprobe was prepared and *in situ* hybridization (ISH) performed. Western blots and immunocytochemistry were used to examine Pi protein and other glial cell markers. The data indicated that Pi protein increased during the first 2 d after withdrawal of the toxicant, when the level of the myelin marker, 2', 3'-cyclic nucleotide-3'-phosphohydrolase, remained minimal. Results of ISH suggested that levels of Pi message in the corpus striatum decreased during cuprizone feeding and began to recover within 2 d after withdrawal of the toxicant. Both microglia and astrocytes appeared during the first week of cuprizone administration and persisted during two to three additional weeks on cuprizone. Reactive astrocytes remained in the tissue for at least 6 wk after cuprizone was withdrawn, while microglia receded within days. The findings suggest that astrocytes continue to express Pi after withdrawal of cuprizone.

Index Entries: Glutathione-*S*-transferase; cuprizone; neurotoxin; gliosis; astrocytes; oligodendrocytes; demyelination; microglia.

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INTRODUCTION

Although successful remyelination in the CNS of humans is quite rare, demyelination can be followed by limited degrees of remyelination in the CNS of some patients with multiple sclerosis. Pathological studies of CNS autopsy tissue suggest that some new oligodendrocytes can be generated and can remyelinate. Glial scarring by astrocytes is another well-known consequence of multiple sclerosis (reviewed by Raine, 1984; Norton, 1996).

Cuprizone (*bis*-cyclohexanone oxalyldihydrazone) intoxication in weanling mice has served as a model for demyelination, followed by remyelination, in the CNS. Blakemore (1972) showed that the primary effect of cuprizone was on the oligodendrocytes, which degenerated, and that other events included the appearance of microglia and astrocytes, followed by demyelination. It is reasonable to propose that knowledge about steps in remyelination after cuprizone would provide clues about how remyelination might be encouraged in other instances of demyelination. We administered cuprizone to young adult mice in order to examine remyelination, with immunoreactivity for the Pi isoform of glutathione-S-transferase (Pi) serving as one of several markers for changes in oligodendrocytes and myelinated tracts (Tansey and Cammer, 1991; Cammer and Zhang, 1993). During the first 4 wk, the numbers of Pi-positive oligodendrocytes fell, and Pi immunoreactivity began to appear in astrocytes. Pi remained in astrocytes for at least 100 d of cuprizone feeding. Another glial cell enzyme, carbonic anhydrase II (CAII), was also expressed in the astrocytes, but declined so that, by the seventh week of cuprizone ingestion, Pi-positive astrocytes outnumbered CAII-positive astrocytes by a factor of two (Cammer and Zhang, 1993).

During short-term cuprizone intoxication in weanling mice, healthy oligodendrocytes appear to be generated while the mice are still consuming the toxicant; however, remyelination cannot take place until the toxicant is withdrawn (Ludwin, 1978). We have not previously reported on the expression of Pi after removal of cuprizone. The present experiments address the changes in expression of Pi shortly after removal of the cuprizone from the diet of the mice and also compare the behavior of microglia and astrocytes during that period of time.

METHODS

As described previously, 6-wk-old male Swiss mice (from Charles River Laboratories, Boston, MA) were administered a diet containing 0.5% (w/w) cuprizone (Sigma, St. Louis, MO) for varying periods of time (Cammer and Zhang, 1993), which will be specified in the text and legends. The normal diet replaced the cuprizone-containing diet for various times thereafter, also to be specified below. To obtain the data shown here, groups of mice received short-term cuprizone (3–4 wk) and were allowed to recover subsequently. Control animals were fed only the normal diet. The feeding

protocol and all the procedures mentioned below were performed in accordance with the guidelines of the AAALAC and had been approved by the Animal Institute at the Albert Einstein College of Medicine.

To obtain brains for quantitation of protein and RNA, mice were sacrificed by ip injection of 100 mg/kg pentobarbital, their heads removed and their forebrains dissected. For preparing the samples for polyacrylamide gel electrophoresis and transfer to nitrocellulose, the forebrains were homogenized in 1% sodium dodecyl sulfate (SDS). Electrophoresis and transfer were performed as described (Towbin, et al., 1979; Tansey et al., 1996). Aliquots of homogenates from each of the animals were placed in wells on triplicate slab gels, with molecular-weight standards on each. Respective sheets were blocked and then immunostained with the following antibodies: rabbit antimouse Pi (from Biotrin, Dublin, Ireland and Cedar Knolls, NJ) at 1:500; mouse antihuman 2′, 3′-cyclic nucleotide-3′-phosphohydrolase (CNP) (from Fisher, Pittsburgh, PA) at 1:100, and mouse anti-glial-fibrillary-acidic protein (GFAP) (from Boehringer-Mannheim, Indianapolis, IN) at 1:100. The secondary antibodies used were alkaline phosphatase conjugated goat antirabbit IgG for Pi or alkaline phosphatase conjugated goat antimouse IgG (from Kirkegaard and Perry, Gaithersburg, MD) for CNP and GFAP. The immunopositive bands were visualized using the nitro blue tetrazolium chloride/5-bromo-4-chloro-3′-indoylphosphate *p*-toluidine salt method. The Shimadzu scanner was used for quantification. An additional antibody, used only for immunocytochemistry, was MAb F4/80. This marker for mouse microglia (Perry and Gordon, 1987) was obtained from Dr. Siamon Gordon, Oxford, UK.

RNA was obtained from weighed forbrains as described and stored at −20°C as an ethanol precipitate (Cammer et al., 1995). The antisense riboprobe to Pi was prepared from cDNA (Dr. I. Listowsky, clone FC13), using the Promega Riboprobe Gemini II in vitro transcription kit and the method of Melton et al. (1984). The DNA was cut with the restriction enzyme EcoRV and electrophoresed; the band was cut out and electroeluted. Runoffs were generated using T_3 polymerase with ^{32}P-CTP for labeling the riboprobe. Northern blots were performed as described (Cammer et al., 1995), using 10 µm of RNA per lane.

Before immunocytochemistry and ISH, animals were administered ip injections of pentobarbital and, while unconscious, perfused through the heart with 4% paraformaldehyde (w/v), 1.4% lysine (w/v), and 0.2% $NaIO_4$ (w/v). The brains were dissected and postfixed overnight in the same fixative at 4°C. Vibratome sections were cut at a thickness of 50 µ. Some were placed on slides and stored at −70°C for subsequent ISH. Some brains were frozen and 10-µ sections prepared and immunostained with 3,3′-diaminobenzidine as color reagent using conventional methods described previously (Cammer et al., 1995).

For ISH, the Pi riboprobe was labeled with ^{35}S-UTP (Amersham, Arlington Heights, IL). ISH methods were obtained from an article by Angerer et al. (1987); our modifications have been described (Tansey et al., 1996).

Table 1
Glial-Cell and Myelin Antigens in Mouse Brains During and After Cuprizone Ingestion (percentages of amounts [per mg protein] in control brains)

Antigens	Cuprizone 27 d	Cuprizone 25 d off 2 d
Pi	80 ± 8	133 ± 8
p	n.s.	$p < 0.01^a$
GFAP	231 ± 18	274 ± 1.4
	$p < 0.002$	$p < 0.002$
CNP	54 ± 10	60 ± 6
	$p < 0.01$	$p < 0.01$

Values are given as ± SD.
p values in table represent comparisons with controls.
$^a p < 0.002$ vs 27 d on cuprizone.
Four control values and four experimental values were used to obtain each parameter.
n.s., not significant.

RESULTS

Western blots were used for comparing the amounts of Pi per mg protein in forebrain homogenates from mice that had ingested cuprizone. The values for Pi at 2 and 4 wk were only slightly lower than the amount of Pi in control forebrains. However, there occurred significant accumulation of Pi between the fourth and seventh weeks on cuprizone ($p \leq 0.002$), such that the level was 106% of normal at 7 wk.

When mice were fed cuprizone for 3–4 wk and the toxicant removed from the diet for 48 h thereafter, an increase in Pi content occurred (Table 1). The astrocytic antigen, GFAP, had increased more than twofold during the 27 d of intoxication and remained high after 25 d of intoxication, followed by 2 d on the normal diet. The myelin- and oligodendrocyte-antigen, CNP, which had decreased, did not change significantly during the two subsequent days on the normal diet (Table 1).

After at least 6 wk, following return to the normal diet, there remained many Pi-positive astrocytes (Fig. 1). Panel A shows an intensely Pi-positive astrocyte (arrowhead) after 15 d of recovery, accompanied by an oligodendrocyte (arrow); panel B, an astrocyte (arrowhead) after 22 d of recovery; and panel C, after 42 d of recovery, when both astrocytes (arrowheads) and oligodendrocytes (arrows) were evident.

An antisense riboprobe for Pi was prepared for performing ISH in the experimental mice. Northern blotting (Fig. 2) showed the validity of using this riboprobe for Pi: at ~800 bp, the size of the Pi message was close to the value of 750 bp reported for the mRNA for Pi in rat (Sug-

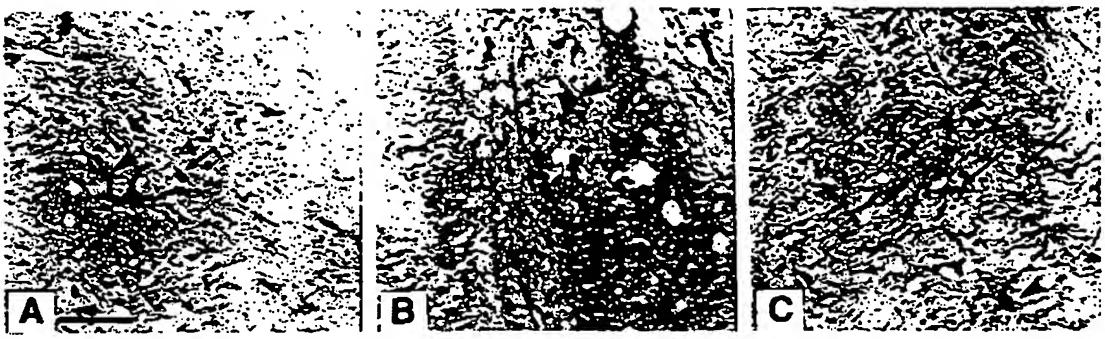

Fig. 1. Cellular localization of Pi in frozen sections after removal of cuprizone from the diet. Mice were fed cuprizone for 28 d, then returned to normal diet for 15 d (A), 22 d (B), or 42 d (C). Arrowheads point to Pi-positive astrocytes and arrows to Pi-positive oligodendrocytes. Bar = 50 μ.

Fig. 2. Northern blot of Pi message in RNA from control mice (lanes 1 and 2) and mice that had been fed cuprizone for 27 d (lanes 3 and 4). Upper marker represents 28S and lower, 18S. The length of the mRNA for Pi is approximately 800 bp.

uoka et al, 1985). The amount of Pi mRNA was significantly reduced after 27 d of cuprizone (lanes 3 and 4).

In tracts of the corpus striatum, there occurred a partial decrease in the density of Pi mRNA signal after 27 d of cuprizone feeding (Fig. 3, panel B), compared to the ISH data from control mice (panel A) and from mice that received cuprizone for 25 d followed by normal food for 2 d

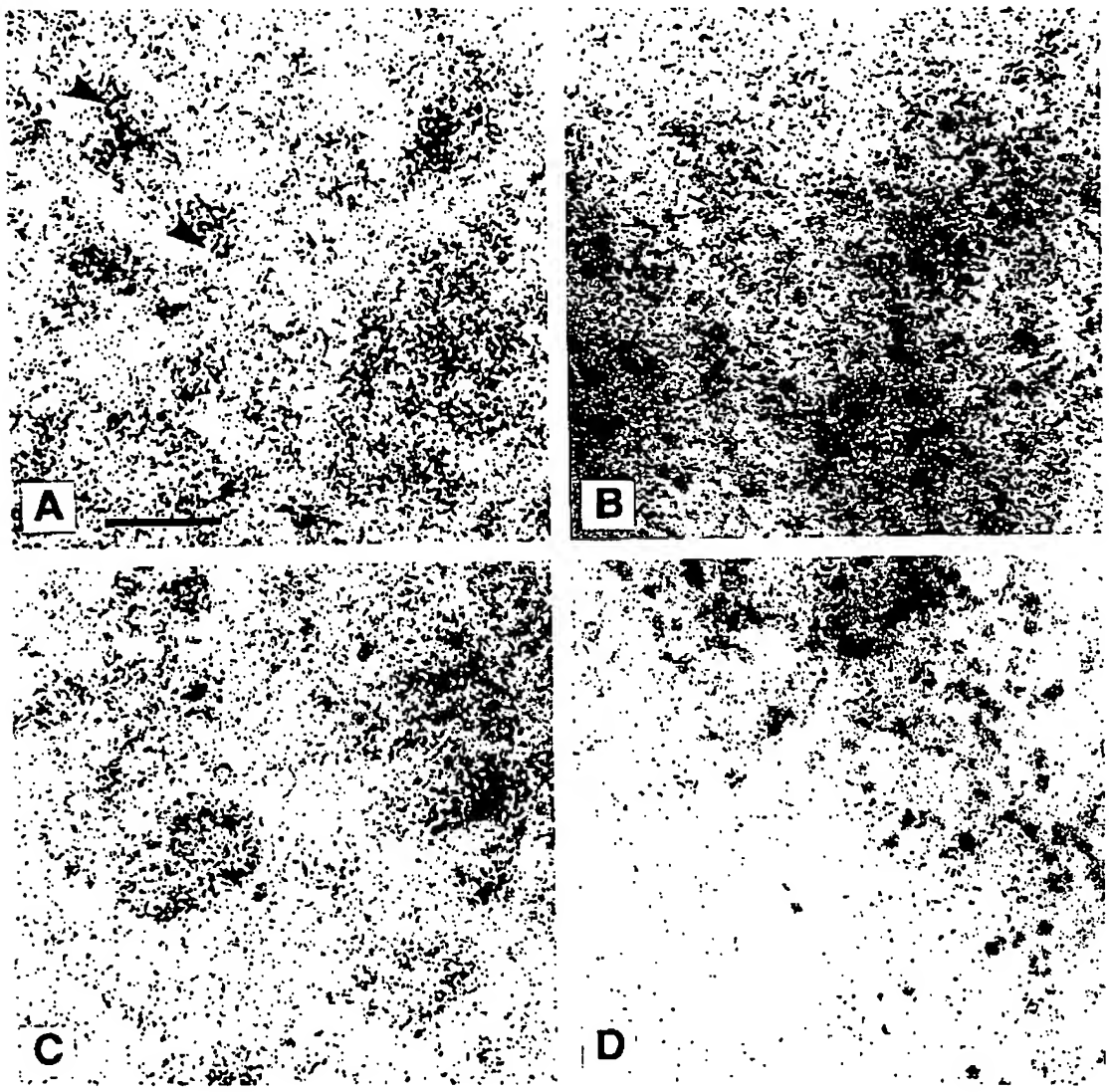

Fig. 3. *In situ* hybridization for Pi mRNA in brains from normal and cuprizone-fed mice. Corpus striatum is shown from control mouse **(A)**; mouse fed cuprizone for 27 d **(B)**; mouse fed cuprizone for 25 d and normal food for two subsequent days **(C)**; and RNAse-pretreated section from control mouse **(D)**. Arrowheads in (A) point to some examples of oligodendrocytes. Bar = 50 μ.

(panel C). Panel D shows a control section that had been treated with RNAse. In the normal corpus striatum, some oligodendrocytes could be discerned (arrows to small round cells in panel A); however, the resolution was sufficient only for observing the tracts but not the specific cellular localization during and after cuprizone feeding (panels B and C).

Macrophages and microglia had been observed during studies of younger mice that had consumed cuprizone (Blakemore, 1972, 1973; Ludwin, 1978, 1980). In young adult mice, microglia and astrocytes appeared early, e.g., 4 and 6 d after starting cuprizone (Fig. 4). In Fig. 5,

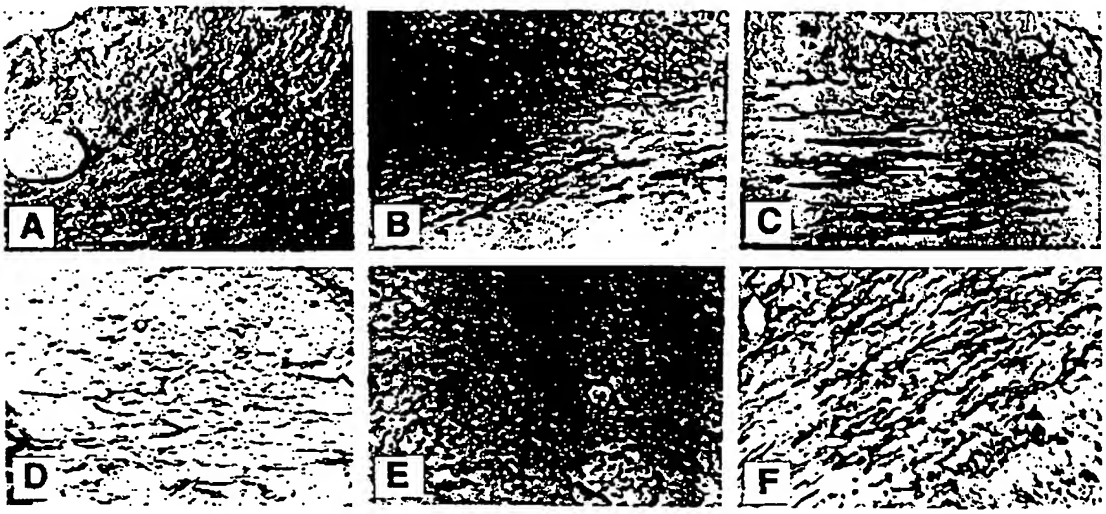

Fig. 4. Microglia (A–C) and reactive astrocytes (D–F) in frozen sections from mouse brains during the first week of cuprizone administration. Microglia were immunostained with MAb F4/80 and astrocytes with anti-GFAP. A–C, F4/80; A, control; B, cuprizone 4 d; C, cuprizone 6 d. D–F, GFAP; D, control; E, cuprizone 4 d; F, cuprizone 6 d. Bar = 50 μ.

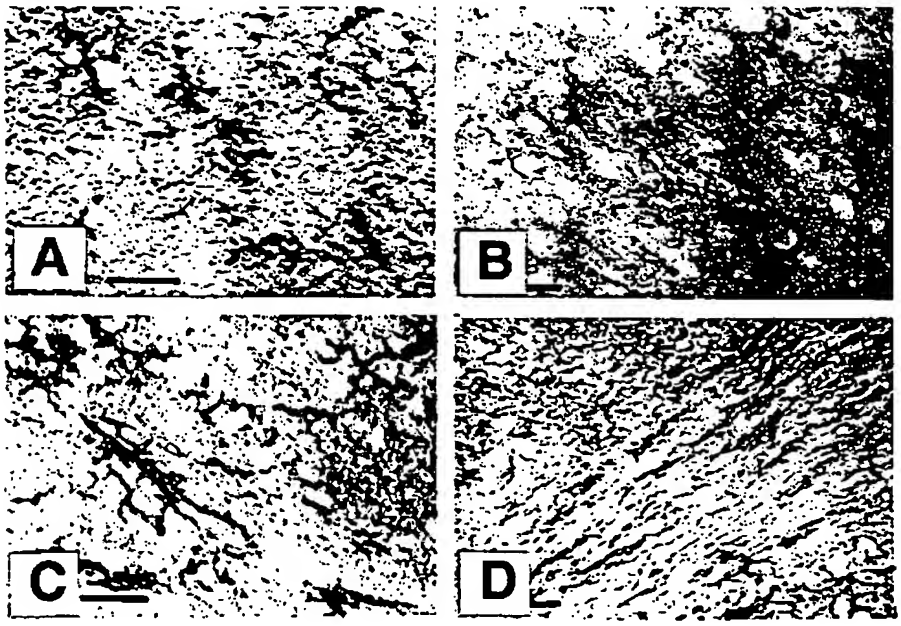

Fig. 5. Microglia in frozen sections from brains of control, cuprizone-fed, and recovering mice. Microglia were immunostained as described in Fig. 4. A, cuprizone 21 d, gray matter; B, cuprizone 21 d, and 2 d off cuprizone, gray matter; C, cuprizone 21 d white matter; D, cuprizone 21 d, and 2 d off cuprizone, white matter. Bar = 25 μ.

microglia are shown in gray matter (panel A) and white matter (panel C) of a mouse that had been consuming cuprizone for 21 d. Panels B and D show that, after 2 d recovery, the microglial reaction had begun to recede.

DISCUSSION

During cuprizone ingestion for ~4 wk, the density of silver grains for Pi mRNA in myelinated tracts in the corpus striatum (in particular) thinned out (Fig. 3, panel B), consistent with the previously observed decrease in immunocytochemical staining for Pi in those tracts (Cammer and Zhang, 1993). There appeared to be a greater loss of Pi mRNA than of Pi protein in the whole forebrain (Table 1 and Fig. 2). Subsequently, only 2 d after return to the normal diet, both Pi protein (Table 1) and the density of the silver grains (Fig. 2) increased, whereas amounts of GFAP and CNP proteins remained at the cuprizone-induced levels (Table 1). Messages for Pi and CAII have now been localized in oligodendrocytes (Fig. 3, panel A; and Tansey et al., 1996). Probably on account of the larger cell volume of the reactive astrocytes, however, silver grains were too widely scattered to provide positive localization in the latter in vivo (panels C and D). Nevertheless, some conclusions are possible. At approximately d 50 of cuprizone intoxication in young adult mice, the numbers of Pi-positive astrocytes reach their maximum and Pi-positive oligodendrocytes their minimum, such that astrocytes account for ~90% of the total Pi-positive glial cells (Cammer and Zhang, 1993). Normally, that percentage is close to zero (Tansey and Cammer, 1991). It is therefore likely that the increase in Pi protein from 80 to 106% of normal ($p < 0.002$) between the fourth and seventh weeks on cuprizone can be attributed to an increase in Pi-positive astrocytes.

It is unfortunate that ISH with a Pi probe lacked the resolution for distinguishing between astrocytes and oligodendrocytes after cuprizone was withdrawn (Fig. 3). Astrocytes did remain Pi-positive during short or long intervals after removal of cuprizone from the diet (Fig. 1). However, Pi-immunopositive oligodendrocytes became evident, as well, in the tissue (Fig. 1). During the 2-d interval, there occurred no significant quantitative changes in the respective glial-cell markers, CNP or GFAP (Table 1). Although the present data do not distinguish in which glial cells (or in both types) Pi is upregulated after withdrawal of cuprizone, it is likely that many are astrocytes.

Experimental allergic encephalomyelitis (EAE) has served as an approximate animal model for inflammatory demyelinating disease in humans (reviewed by Raine, 1984). Gliosis occurs rapidly during the course of an acute episode of EAE in the Lewis rat, and GFAP and mRNA for GFAP persist for at least 9 wk after the initial sensitization (Smith et al., 1983; Aquino et al., 1988, 1990). In contrast to the gliosis, little inflammation remains at the end of that time (Aquino et al., 1988). Long-term persistence of reactive astrocytes also has been observed previously in the brains of neurological mutant mice and of human patients with the hereditary disease adrenoleukodystrophy; whereas numbers of reactive astrocytes decrease more rapidly after gliosis due to injury or to toxicants that damage neurons (reviewed by Norton et al., 1992). The observations of transient

inflammatory cells (Figs. 4 and 5) and long-term persistence of reactive gliosis after discontinuation of the oligodendrocytic toxicant, cuprizone, in young adult mice (Fig. 1) ressemble EAE more than they do the events subsequent to physical injury or toxic attack on neurons. In cuprizone intoxication, as in EAE (Smith et al., 1983), inflammation and gliosis began at about the same times, which are in the early stages (Fig. 5).

The question of whether astrocytes facilitate or hinder remyelination has not been settled and may have different answers depending on the circumstances. During cuprizone intoxication, some astrocytes, as well as many microglia, have been observed removing products of myelin breakdown (Blakemore, 1973; Ludwin, 1978). Astrocytes appear able also to protect oligodendrocytes and/or encourage remyelination after other instances of demyelination (Wu and Raine, 1992; Franklin et al., 1993). It is possible that some of the Pi in astrocytes results from the uptake of shrunken oligodendrocytes and oligodendroglial membranes. If so, that would account for the minor decrease in Pi protein (Table 1) in the presence of a major change in Pi message (Figs. 2 and 3). Future studies should address this and other questions brought up by the present data—for example whether Pi continues to be synthesized after the toxicant is withdrawn and/or whether Pi protein turns over more slowly in astrocytes than in oligodendrocytes. The latter might be the case if Pi were situated inside bundles of intermediate filaments. Finally, toxicants or reactive oxidation products may be conjugated to Pi or conjugated by Pi to glutathione, and the conjugates stored in reactive astrocytes where they would be sequestered from oligodendrocytes and neurons.

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